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Specific thiol determination by micellar electrokinetic chromatography and on-column detection reaction with 2,2′-dipyridyldisulfide

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Abstract

A new method for specific determination of glutathione using micellar electrokinetic chromatography and on-column reaction with 2,2′-dipyridyldisulfide is described. 2,2′-Dipyridyldisulfide and a sample of glutathione are injected consecutively into the capillary as two discrete plugs separated with a short plug of background electrolyte. Due to the differences in the mobilities of the 2,2′-dipyridyldisulfide and glutathione, on-column mixing and reaction occur. Glutathione is in this reaction quantitatively transformed into a mixed disulfide concomitantly with formation of an equimolar amount of the 2-thiopyridone which is further separated by micellar electrokinetic chromatography and determined spectrophotometrically at 343 nm. The concentration of glutathione is thus estimated indirectly from the result of 2-thiopyridone determination. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Detection, electrophoresis; 2,2′-Dipyridyldisulfide; Thiols; Glutathione; Sulfur compounds

1. Introduction

Capillary zone electrophoresis (CZE) is a widely used analytical technique. It has several advantages – high efficiency, short time of analysis, small amounts of sample and buffers and finally small amount of waste. The narrow diameter of separation columns that permits the advantages mentioned above is also responsible for the major limitation of the technique. Although mass sensitivity is extremely high because of the very small detection volume, the concentration sensitivity, especially in the case of UV absorbance detection, is generally several times lower than that in high-performance liquid chromatography (HPLC).

To improve sensitivity, derivatization for spectrophotometric, fluorescence or electrochemical detection is utilized and performed either in the pre-, post- or on-column mode. However, the pre- and post-column derivatization procedures have several disadvantages. The pre-column derivatization method requires an additional sample manipulation while special equipment has to be connected to the standard CZE system when the post-column derivatization is used. Moreover, derivatization performed either in the pre- or post-column mode commonly implies dilution or waste of a portion of the original sample. The mentioned disadvantages were eliminated in the recently developed on-column approach [1]. In this set-up a derivatization reaction is performed during the electrophoretic separation. In principle, the front end of the capillary is used as the reaction chamber. The analyte and the reagent are injected separately and are mixed because of their different electrophoretic mobilities. This approach is especially suitable for the determination of the

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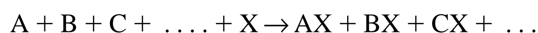
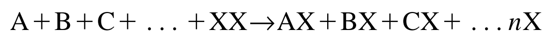
analytes presented in extremely small sample volumes such as in a single cell analysis [2].

Various on-column reactions have been demonstrated so far however only for derivatization of the amino-group containing compounds such as amino acids [3–9], amines [10,11], diamines [10,11], polyamines [11], peptides [2] and proteins [12]. In all these applications the simplified derivatization scheme is the same. The detector nonresponding analytes A, B and C are converted into the derivatives AX, BX and CX that have enhanced detector response properties, in the chemical reaction with the derivatization reagent X:



$$A + B + C + \ldots + X \rightarrow AX + BX + CX + \ldots$$

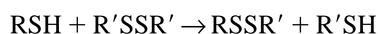
In this paper a new type of on-column reaction detection is reported. The analytes A, B and C react with the reagent XX forming the derivatives AX, BX and CX, and a reaction by-product X. In contrast to the methods mentioned above, this highly absorbing by-product X is used for the detection:



$$A + B + C + \ldots + XX \rightarrow AX + BX + CX + \ldots nX$$

Considering that the by-product X is identical for all analytes, the separation of the analytes has to be performed before the reaction.

As an example of the utility of this concept the thiol–disulfide exchange reaction was used for detection of thiols. It is the reaction between an aliphatic thiol RSH and a so called reactive disulfide R′SSR′. When the thiol compound is allowed to react with the disulfide, which is present in excess, the mixed disulfide R′SSR and the corresponding thiol R′SH are formed:



$$RSH + R'SSR' \rightarrow RSSR' + R'SH$$

These reactions have been found to be rapid and quantitative at room temperature, because the corresponding thiols R′SH are stabilized either by resonance or by thiol–thione tautomerism. This fact also causes a large shift of the absorption maxima toward longer wavelengths.

The properties of reactive disulfides [2,2′-dipyridyldisulfide, (5-carboxy-2-pyridyl)disulfide, 5,5′-dithiobis-(2-nitrobenzoic acid), etc.] have been used in a number of applications – for spectrophotometric determination of thiol groups in proteins [13,14], as activation reagent in covalent chromatography [15], for pre- and post-column derivatization of thiols in HPLC [16,17] or for pre-column derivatization of thiols in CZE [18]. The present paper describes the usage of the on-column reaction of 2,2′-dipyridyldisulfide for the specific detection of thiols (Fig. 1) during micellar electrokinetic chromatography (MEKC). There were several reasons for choosing 2,2′-dipyridyldisulfide for this purpose: (i) this compound is a neutral molecule and its mobility in MEKC can be easily manipulated, (ii) the separation window of the method [the time window between electroosmotic flow (EOF) marker and the zone of 2,2′-dipyridyldisulfide] is large because of the big differences in the hydrophobicity of 2,2′-dipyridyldisulfide and separated thiols and (iii) the spectra of 2,2′-dipyridyldisulfide and its reaction products are rather different. Although this system has been demonstrated specifically for the determination of glutathione, it can be applied for determination of the other thiols after modification of separation conditions.

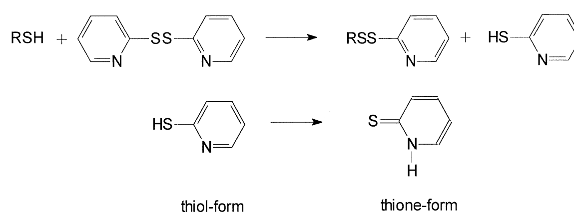


Fig. 1. Reaction of 2,2′-dipyridyldisulfide with thiol RSH.

2. Experimental

2.1. Materials and reagents

2,2′-Dipyridyldisulfide (DPDS), dithiothreitol (DTT), reduced (GSH) and oxidized (GSSG) glutathione, homocysteine (HCys), *N*-acetylcysteine (NACys) and cysteine (Cys) were obtained from Sigma (St. Louis, MO, USA). All other chemicals and solvents were of analytical-reagent grade, supplied from Fluka (Buchs, Switzerland). Standards of thiols were prepared in 50 m*M* phosphate buffer (pH 7.5) containing 1 m*M* EDTA fresh each day. DPDS

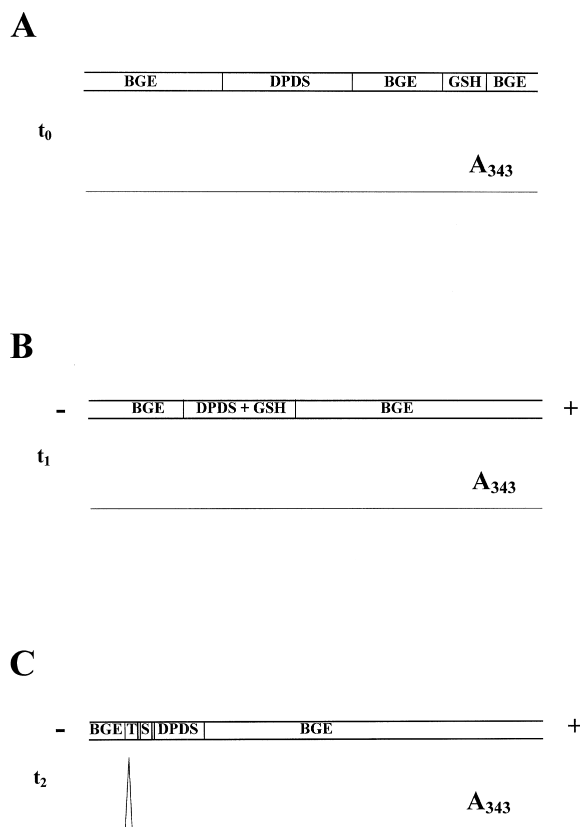


Fig. 2. Schematic representation of on-column detection procedure. BGE, Background electrolyte; DPDS, 2,2′-dipyridyldisulfide; GSH, glutathione; T, 2-thiopyridone; S, corresponding mixed disulfide.

was prepared as 0.1 *M* stock solution in acetonitrile, that was diluted 20 times with the background electrolyte before the use. All solutions were prepared with Milli-Q Academic water (Millipore, Milford, MA, USA) and filtered through a 0.45-μm membrane filter.

2.2. Instrumentation

A Hewlett-Packard ^{3D}Capillary Electrophoresis system (Waldbronn, Germany) with a diode-array UV–Vis detector was used to carry out all separations. Data were collected on an HP Vectra VL 5 166 MHz personal computer using the Hewlett-Packard ^{3D}CE ChemStation software. A Hewlett-Packard extended light path capillary [64.0 cm (58.8 cm effective length)×50 μm I.D.] was used for all analyses. The capillary was washed with 0.1 *M* NaOH for 1 min, deionized water for 1 min and buffer for 3 min before each run and washed with deionized water for 3 min after each run. The on-column detection reaction was performed by injection the DPDS solution, the background electrolyte, a sample and finally the background electrolyte consecutively for a specified time as given in the text below. Separations were performed at 28 kV (positive polarity). Samples were detected using a diode-array detector at 200 nm and 343 nm with a bandwidth 10 nm. Spectra were also collected during the runs for peak identification. The purity of the peaks was checked by the spectral utility of the ChemStation software. In addition, the identity of GSH peak was further supported by spiking samples with the standard.

2.3. Sample preparation

2.3.1. GSSG reduction [19]

A 0.1-ml volume of 50 m*M* DTT was added to 0.9 ml of 200 μ*M* GSSG standard. The solution was kept at room temperature for 30 min to allow the total reduction and then used for analysis.

2.3.2. Whole blood sample [20]

Fresh blood samples were collected from healthy volunteers to the anticoagulant heparin tubes. A 0.450-ml volume of blood was pipetted into a 1.5-ml centrifuge tube and 0.05 ml of 50% sulfosalicylic acid solution containing 1 m*M* EDTA was added in an ice bath. The tube was immediately capped, vortex-mixed for 5 min and centrifuged at 15 000 rpm for 5 min. The supernatant was filtered through a 0.45-μm filter and used for analysis.

2.3.3. Erythrocytes sample [18]

Blood samples were centrifuged at 2500 rpm for 5 min and the plasma layer was removed. The erythrocytes were then washed twice with equal volumes of isotonic saline solution (0.9% NaCl). The erythrocytes were lysed by freezing in liquid nitrogen followed by thawing in a water bath at 60°C. This procedure was repeated three times to ensure complete cell lysis. A 1.0-ml volume of the lysed erythrocytes was added to 1.0 ml of 0.1 *M* trichloroacetic acid solution containing 1 m*M* EDTA. The

sample was vortex-mixed for 5 min and centrifuged at 3500 rpm for 5 min to remove protein precipitate. The supernatant was filtered through a 0.45-μm filter and used for analysis.

3. Results and discussion

The principle of on-column detection reaction as carried out in this study is schematically described in

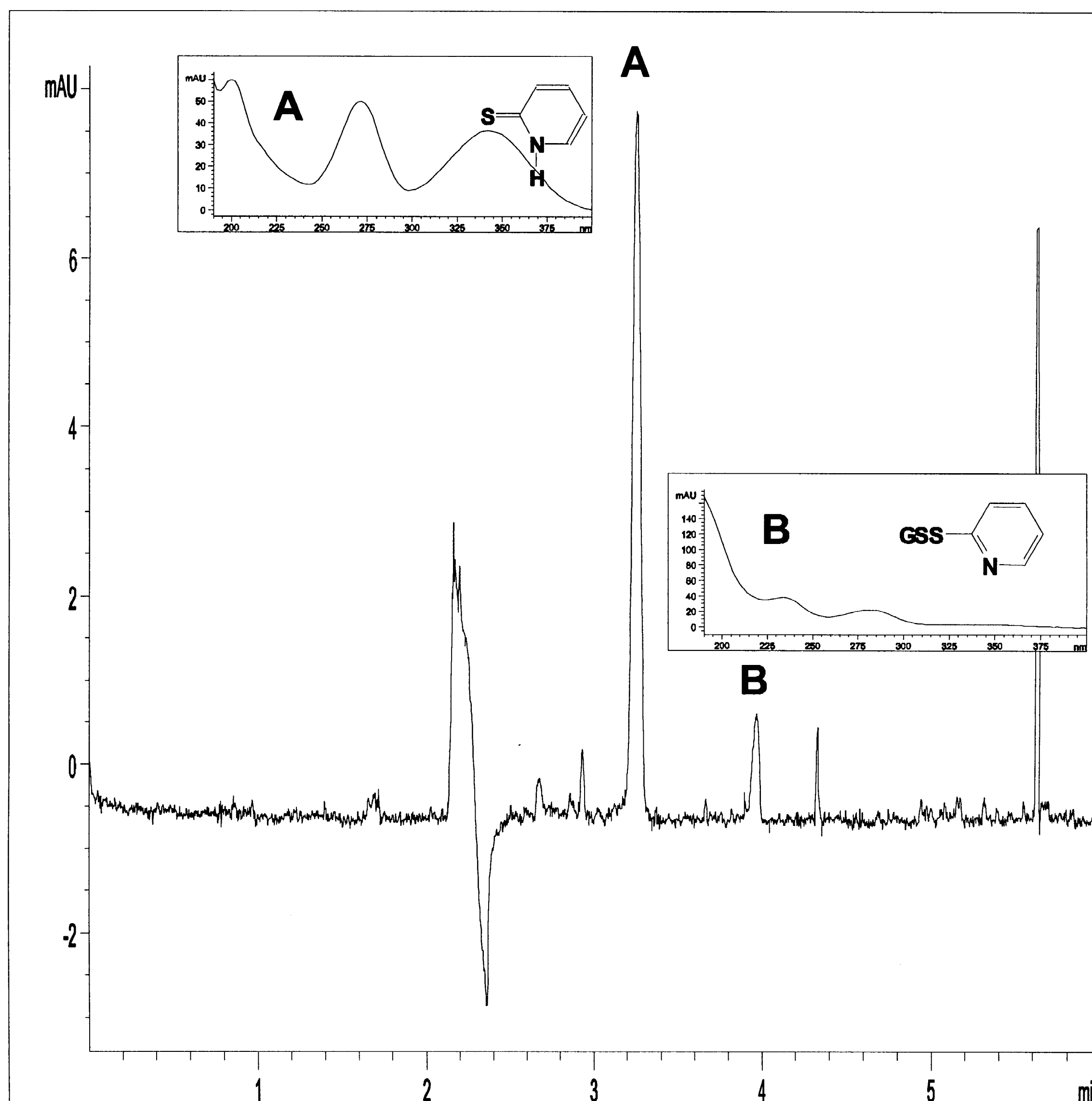


Fig. 3. Electropherogram of 200 μ*M* GSH standard. Peak identification: (A) 2-thiopyridone, (B) mixed disulfide between glutathione and 2-pyridylsulfide. Insert: the spectra of the peaks taken using diode array detection. Separation conditions: background electrolyte, 50 m*M* SDS in 50 m*M* phosphate buffer (pH 7.5); separation voltage, 28 kV (positive polarity); temperature of capillary, 20°C; detection at 343 nm and sampling, 5 m*M* DPDS, 50 mbar for 20 s; the background electrolyte, 50 mbar for 120 s; the sample, 50 mbar for 6 s; the background electrolyte, 50 mbar for 2 s.

Fig. 2. DPDS and a sample of GSH are injected consecutively in the capillary as two discrete plugs separated with short plug of background electrolyte (Fig. 2A). Due to the differences in the mobilities of the DPDS and GSH, the zone of GSH is approaching to the zone of DPDS and on-column mixing and reaction occur (Fig. 2B). GSH is thus quantitatively transformed into a mixed disulfide concomitantly with formation of the equimolar amount of 2-thiopyridone which is further separated by MEKC and detected spectrophotometrically at 343 nm (Fig. 2C).

Before the application of on-column reaction in MEKC possible dependence of reaction chemistry on the presence of the surfactant in separation buffer matrix has been investigated. The reaction rate and yield for the reaction of DPDS with GSH were examined both in 50 m*M* phosphate buffer (pH 7) prepared by mixing of 50 m*M* solution of monosodium phosphate and 50 m*M* solution of disodium phosphate, respectively in the same buffer containing 50 m*M* SDS, which is the sufficient concentration for separation of common endogenous and exogenous thiols by MEKC (data not shown). The reactions were followed by measuring the increase in absorbance at 343 nm as a function of time. No differences in the reaction rates and in the yields of 2-thiopyridone were observed for this reaction in the presence of sodium dodecyl sulfate (SDS) micelles compared with a pure phosphate buffer.

In order to determine GSH with the highest sensitivity the reaction/separation parameters – pH of the background electrolyte in the range 6.0–9.5; concentration of the background electrolyte in the

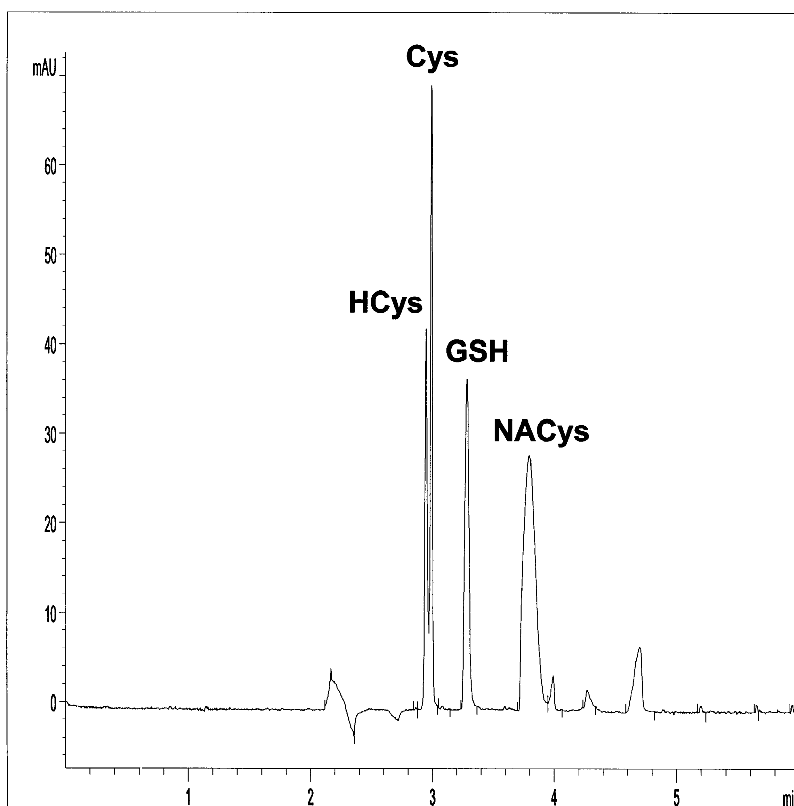


Fig. 4. Electropherogram of the mixture of thiols. HCys, homocysteine (1 m*M*), Cys, cysteine (1 m*M*), GSH, glutathione (1 m*M*) and NACys, *N*-acetylcysteine (2 m*M*). Other conditions as in Fig. 3.

range 20–100 m*M* and the temperature of the capillary in the range 20–50°C were optimized to give the highest yield of 2-thiopyridone. The optimization was performed using the on-column approach because all these parameters had double effect on the on-column reaction. They influenced not only on the reaction itself but also on the reaction time due to the changes of the mobilities of DPDS and GSH. The effect of the size of reagent plug on the yield of 2-thiopyridone was also studied. As mentioned above GSH has to be separated from other thiols before the reaction with DPDS. To achieve the separation, a short plug of the background electrolyte was added between the reagent and sample plugs and its length was optimized to get suitable resolution.

Fig. 3 shows a electropherogram of 200 μ*M* GSH standard under the optimal conditions of the background electrolyte 50 m*M* SDS in 50 m*M* phosphate buffer (pH 7.5); separation voltage 28 kV (positive polarity); temperature of capillary 20°C; detection at 343 nm and sampling parameters: 5 m*M* DPDS, 50 mbar for 20 s; the background electrolyte, 50 mbar for 120 s; the sample, 50 mbar for 6 s; the background electrolyte, 50 mbar for 2 s in described order.

The dominant peak at 3.2 min is 2-thiopyridone with an absorption maximum at 343 nm formed in reaction of DPDS with GSH. The insignificant nointerfering peak at 3.9 min is due the corresponding mixed disulfide that slightly absorbs at this wavelength (see the spectra in Fig. 3). A typical electropherogram of the mixture of common endogenous and exogenous thiols is shown in Fig. 4. It is important to emphasize, that the shown peaks correspond to 2-thiopyridone formed in reaction of DPDS with given thiol. As can be seen, a good resolution of the peak corresponding to GSH from the peaks of the other thiols was achieved.

The quantitative parameters of the developed method were estimated at the optimal conditions. All

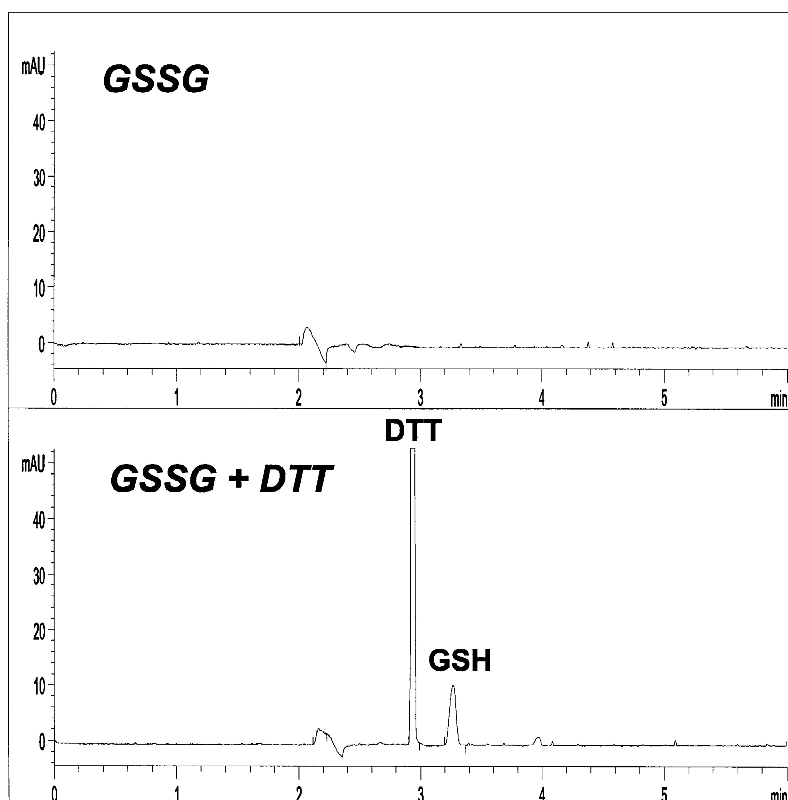


Fig. 5. Electropherograms of 200 μ*M* GSSG standard before and after the reduction with DTT. Other conditions as in Fig. 3.

measurements were performed with GSH standards containing 5 m*M* DTT to prevent the oxidation of GSH. The calibration graph for the peak area was linear over the range of 0.05–2 m*M* of GSH with correlation coefficient better then 0.999. The detection limit was in the range 5 μ*M* at a signal-to-noise ratio of 3. The sensitivity of the method is thus comparable with the method using direct UV detection at very low wavelengths, 185–190 nm [21], at which many of endogenous compounds would be interfering or with the method using 5,5′-dithiobis-(2-nitrobenzoic acid) for the pre-column derivatization [18]. Several methods using fluorescence derivatization with lower detection limits have also been reported [22–24] however they need more special equipment compared to the CZE system used in the method described here.

The results of replicated analyses ($n=10$) showed good reproducibility obtained for peak area (<1.2%) and excellent reproducibility obtained for migration time (<0.6%). The recoveries were tested by the analyses ($n=10$) of the samples spiked with known amounts of GSH. The recovery of GSH by the MEKC method described was estimated to be between 94 and 107%.

The possibility of using this method for determination of oxidized form of glutathione (GSSG) was also tested. As in thiol exchange reaction only reduced form of glutathione is active, it is necessary to perform the reduction of GSSG with excess of DTT. The concentration of GSSG then can be calculated indirectly from the results of GSH determinations before and after reduction. Moreover, no interference of DTT was observed (Fig. 5).

In order to demonstrate the application of the developed method for determination of GSH in a biological matrix, the concentration of reduced GSH in whole human blood was determined. Representa-

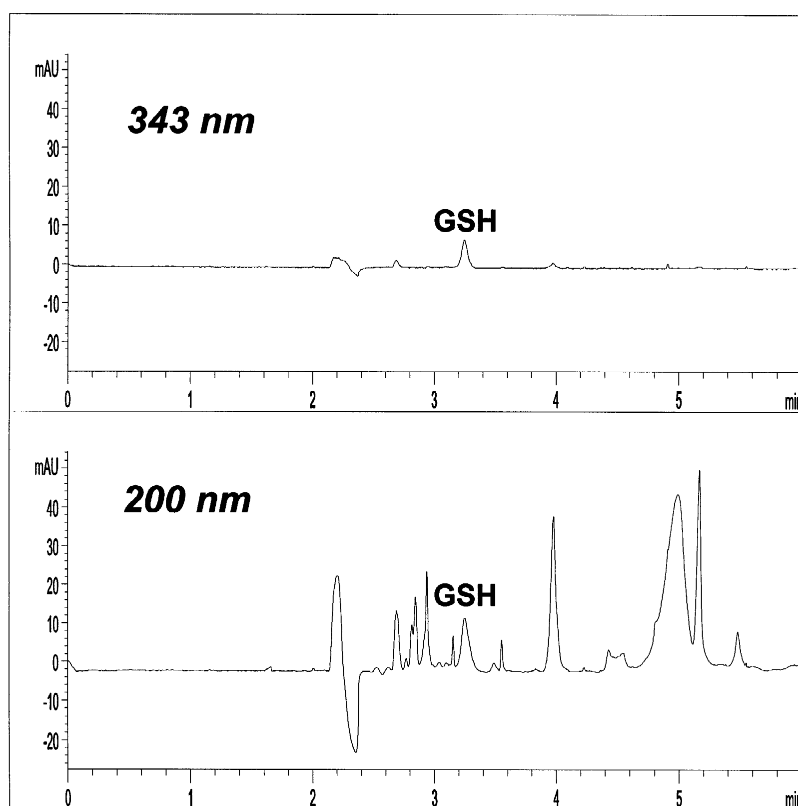


Fig. 6. Electropherograms from the determination of GSH in whole human plasma detected at 343 nm and 200 nm. Other conditions as in Fig. 3.

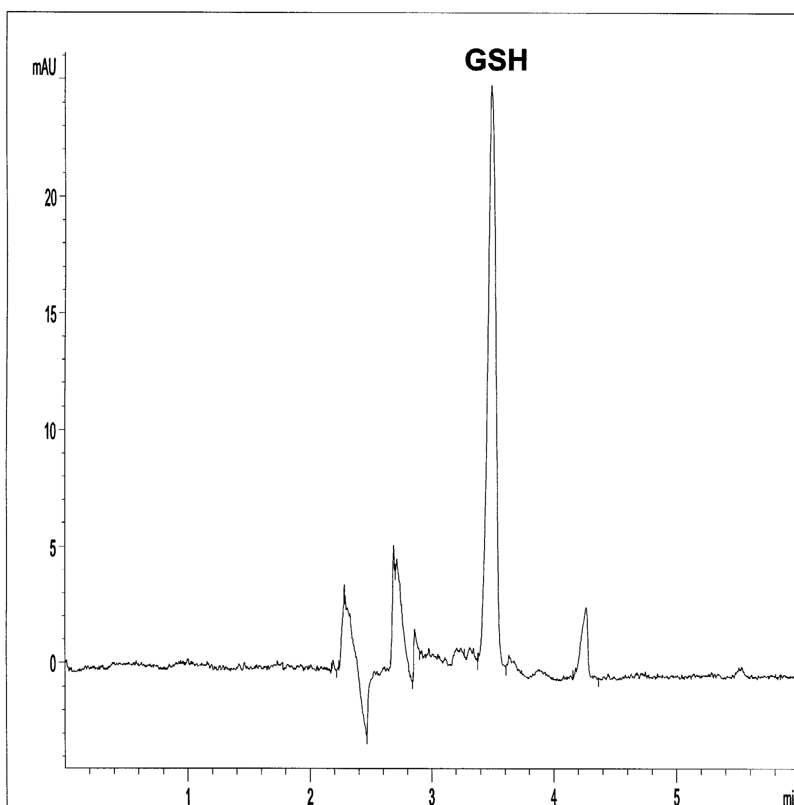


Fig. 7. Electropherogram from the determination of GSH in human erythrocytes. Other conditions as in Fig. 3.

tive electropherograms are shown in Fig. 6. The concentration of GSH found was 645±25 μ*M*. This figure also shows the specificity of the detection at 343 nm in comparison with the same electropherogram detected at 200 nm. As a second typical application we used this method for determination of reduced GSH in human erythrocytes (Fig. 7). The estimated erythrocyte GSH concentration of 2.6±0.08 m*M* is in a good agreement with published values between 2 to 3 m*M* determined using other techniques [18,25,26].

4. Conclusion

A new method for specific determination of glutathione using MEKC and a on-column reaction with 2,2′-dipyridyldisulfide was developed. The method is relatively rapid and simple, offering detection limit below 5 μ*M*. Its sensitivity thus cover the concentration range at which GSH is present in most of biological samples. Moreover the whole procedure can be easily automated. As the reaction is carried out inside the capillary, the method has the potential for analyzing samples presented in extremely small volumes such as for a single cell analysis or for microdialysis samples.

Acknowledgements

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